Cytokine-mediated signals as targets for treatment of rheumatoid arthritis: a JAK inhibitor *in vitro* and *in vivo*

Multiple cytokines play a pivotal role in the pathogenesis of rheumatoid arthritis (RA). For this to happen, the appropriate intracellular signaling pathways must be activated via cytokine receptors on the cell surface. Among them, members of JAK family are essential for the signaling pathways of various cytokines and are implicated in the pathogenesis of RA. An orally available JAK3 inhibitor tofacitinib (CP-690, 550) is currently in clinical trials for RA with satisfactory effects and acceptable safety results. Our *in vitro* experiments have indicated that the inhibition effect could be mediated through the suppression of IL-17 and IFN-g production and proliferation of $CD4^+$ T cells. Here, we document the *in vitro*, *ex vivo* and *in vivo* effects of a JAK inhibitor for the treatment of RA.

KEYWORDS: IL-6 ■ JAK ■ rheumatoid arthritis ■ signal ■ STAT ■ TNF ■ treatment

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Signaling in rheumatoid arthritis

Various intercellular signaling plays a pivotal role in the processes of multiple inflammatory diseases, some of which are mediated by soluble ligands such as cytokines and growth factors, and others are mediated by cognate interactions through costimulatory molecules and adhesion molecules. Rheumatoid arthritis (RA) is a representative autoimmune disease characterized with systemic, chronic and destructive inflammatory synovitis and multiple organ manifestations that cause severe disability and mortality in patients. The importance of inflammatory cytokines in the pathogenesis of RA has become apparent from the clinical efficacies of biological agents targeting TNF and IL-6 [1–3]. For such cytokines to exert their biological activities, the appropriate intracellular signaling pathways must be activated by the engagement of their specific receptors on the cell surface, that is outside to in signaling. The intracellular signals are represented by the following pathways:

- Phosphorylation of protein kinase such as serine/threonine kinase, tyrosine kinase and MAP kinase kinase;
- GTP-binding proteins including small G-protein such as Rho and Ras and heterotrimeric G-protein consisting of Gα, Gβ, Gγ;
- Second messengers such as cyclic AMP and GMP;
- Protease activating apoptotic-related proteins such as caspase; and
- Ubiquitination (Figure 1).

The transduction of these intracellular signals leads to various cellular functions through directly activating, sequentially activating or regulating one another.

Among phosphorylated kinase proteins, more than 99% are serine/threonine kinases in physiological and normal immune systems. On the other hand, the tyrosine kinase is the first intracellular signaling molecule to be phosphorylated following receptor binding in a cytokine response and is involved in fundamental functions such as cell proliferation, differentiation and adhesion in various pathological processes including inflammation and cancer. Therefore, many investigators have shed light on tyrosine kinases as the target for the treatment of various diseases. More than 90 genes encoding tyrosine kinases have been identified from human genome-wide studies and 14 tyrosine kinases are known to be involved in RA [4].

JAK in RA

Of the tyrosine kinases, the JAK family, consisting of JAK1, JAK2, JAK3 and Tyk2, has gathered particular attention since JAKs are essential for the signaling pathways of various cytokines and are implicated in the pathogenesis of RA (Figure 2) [5–9]. After the engagement of homodimeric or heterodimeric receptors, which are constitutively bound to JAKs, JAKs are activated by a conformational change in the receptor that allows trans- and/or auto-phosphorylation of the two bound JAKs. These in turn phosphorylate the cytokine receptors. STAT proteins bind the phosphorylated receptor chains, which allow the JAKs to phosphorylate

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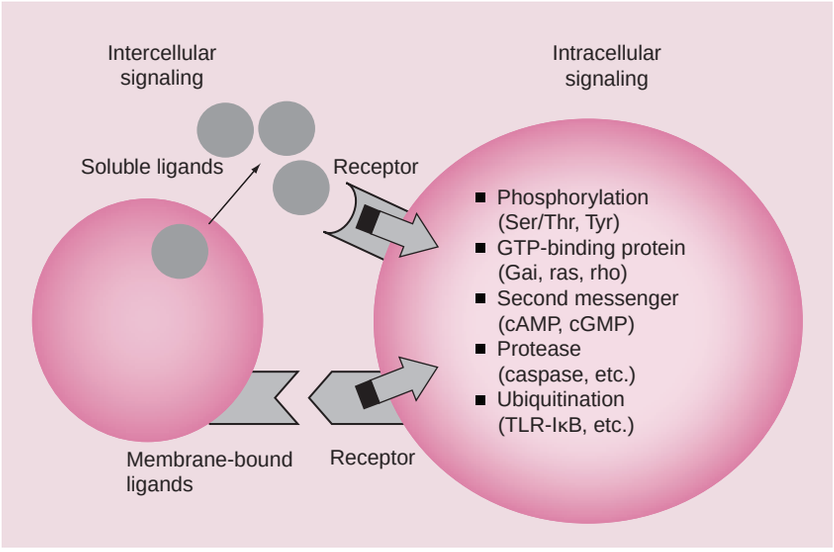

Figure 1. Intercellular and intracellular signaling. The appropriate intracellular signaling pathways must be activated via their specific receptors on the cell surface, that is outside to in signaling.

the STATs. Phosphorylated STATs form dimers and translocate into the nucleus, where they regulate gene expression. Thus, the JAK–STAT pathway regulates multiple immune functions. For instance, different STATs are involved in differential cytokine production from $CD4^+$ T cell subsets: STAT1 and STAT4 mainly induce IFN-γ from Th1, STAT6 induces IL-4 from Th2, STAT5 induses TGF-β from regulatory T cells (Treg) and STAT3 induces IL-17 from Th17 (Figure 3).

JAK3 expression is essentially limited to lymphocytes and constitutively binds to the common γ-chain which is a common receptor subunit for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, the deficiency or dysfunction of JAK3 leads to severe combined immunodeficiency both in humans and mice. Thus, numbers of tyrosine kinase inhibitors have recently been evaluated in clinical trials, selective inhibition of JAK3 was considered as a potential target in the treatment of RA without affecting other organ systems.

Based on these backgrounds, an orally available JAK inhibitor, tofacitinib (CP-690, 550), was developed with expectations to become a new immunosuppressant with a few side effects [10,11]. Tofacitinib dose-dependently improved end points of both, murine collagen-induced arthritis and rat adjuvant-induced arthritis. Tofacitinib was also demonstrated to highly suppress JAK3 with low concentration with a few side effects in a graft versus host disease experiment [10–12]. Furthermore, tofacitinib is currently in clinical trials for RA with satisfactory effects and acceptable safety results. However, the mode of action of tofacitinib in patients with RA remains unclear. Here, we document the *in vitro*, *ex vivo* and *in vivo* effects of a JAK inhibitor for the treatment of RA.

In vitro effects of a JAK inhibitor in RA

JAKs are essential for the signaling pathways of various cytokines and are implicated in the

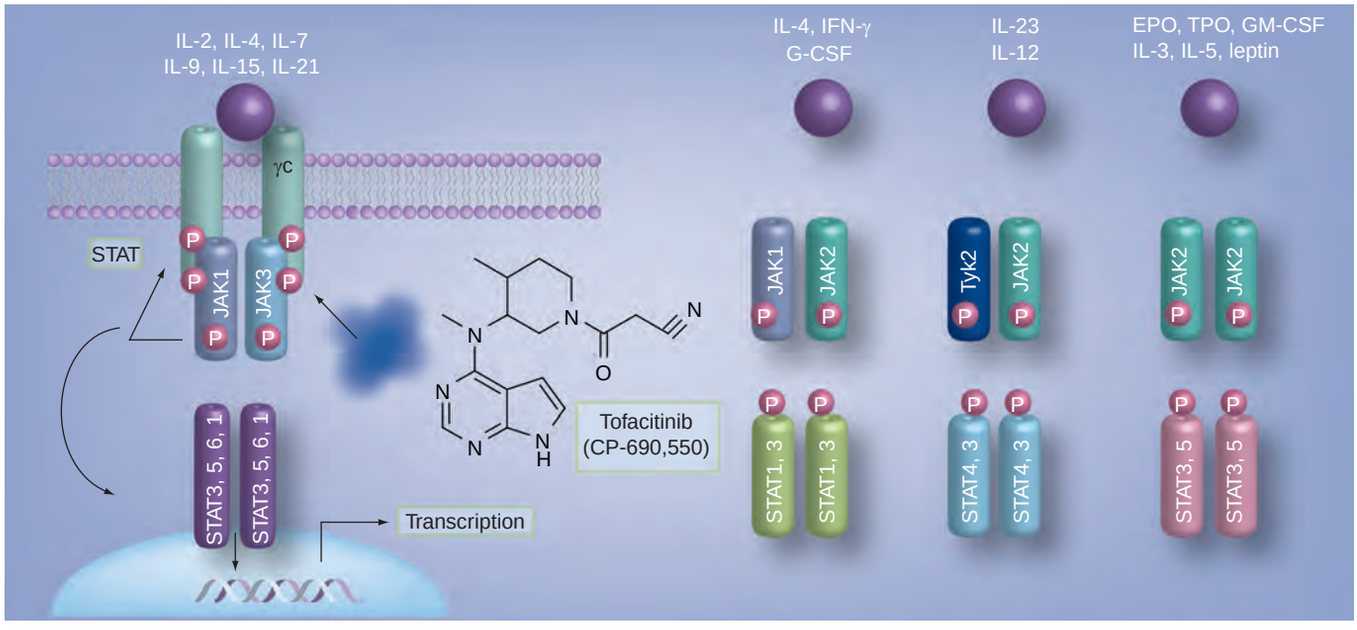

Figure 2. The JAK–STAT signaling pathway. JAK3 expression is essentially limited to hematopoietic cells and constitutively binds to the g–c chain, which is a common receptor subunit for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21.
EPO: Erythropoietin; G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; P: Phosphorylation; TPO: Thrombopoietin.

pathogenesis of RA. Walker *et al.* reported that JAK3, STAT1, STAT4 and STAT6 were highly expressed in synovium in patients with RA, whereas the expression was scarce in synovium in normal volunteers and patients with osteoarthritis and spondyloarthropathy [13]. According to the important role of JAK3 in lymphocyte development, differentiation and proliferation, we assessed the effects of tofacitinib on $CD4^+$ T cells at the local inflammatory sites in patients with RA. Human synovial tissues were obtained from patients with RA undergoing joint replacement surgery at our university. After the tissue was digested with collagenase and dispase and was filtrated, $CD4^+$ T cells and $CD14^+$ cells were isolated by negative selection using a magnetic cell separation system. The proliferation of $CD4^+$ synovial T cells in RA patients stimulated with anti-CD3 and anti-CD28 (anti-CD3/28) antibodies was inhibited by tofacitinib in a dose-dependent manner. Furthermore, treatment of synovial $CD4^+$ T cells with tofacitinib inhibited the production of IL-17 and IFN-γ in a dose-dependent manner, but had no effect on IL-6 and IL-8 production. However, $CD14^+$ monocytes and synovial fibroblasts isolated from synovium in patients with RA were not affected by tofacitinib [MAESHIMA K *ET AL.*, UNPUBLISHED DATA]. Our results suggested that the effects of tofacitinib in RA are mediated through the suppression of IL-17 and IFN-γ production and proliferation of $CD4^+$ T cells without affecting synovial fibroblasts and monocytes. Since IFN-γ and IL-17 are produced by Th1 and Th17 cells, respectively, and are important drivers of destructive arthritis in mice and humans, JAK3 in $CD4^+$ T cells and presumably Th1 and Th17 cells, play a crucial role in rheumatoid synovitis (FIGURE 3).

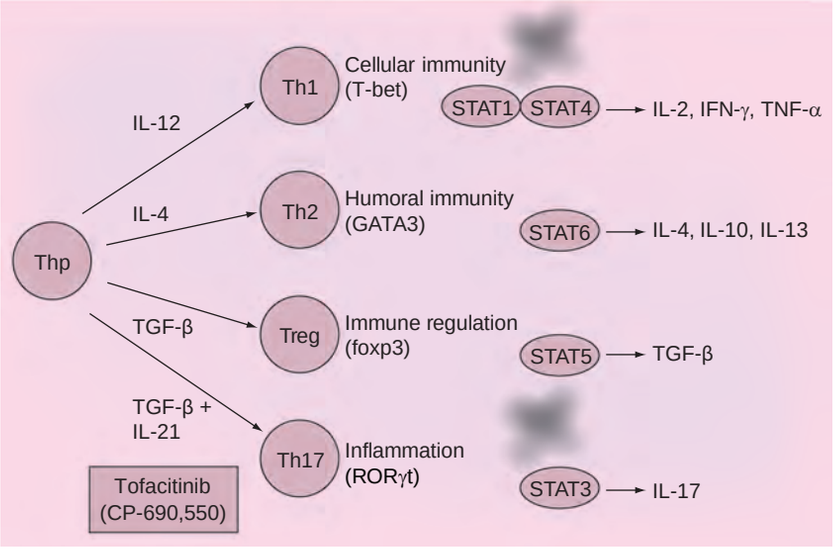

Figure 3. The JAK–STAT signaling pathway in T-cell subsets. Effects of tofacitinib in rheumatoid arthritis are mediated through the suppression of IL-17 and IFN-γ production from $CD4^+$ T cells.

Ex vivo effects of a JAK inhibitor in RA

Next we conducted a treatment study in the severe combined immune deficiency (SCID)-HuRAg mice, an RA animal model utilizing SCID mice implanted with synovium and cartilage donated from patients with RA. Briefly, male SCID mice (C. B-17/lcr), 6–8 weeks old, were housed in specific pathogen-free conditions at our university animal center. Synovial tissue and articular cartilage were transplanted onto the back of the SCID mice. A week after implantation, 0, 1.5 or 15 mg/kg/day of tofacitinib dissolved in polyethylene glycol was administered continuously via Alzet osmotic mini pumps implanted subcutaneously on the backs of the mice. Treatment of SCID-HuRAg mice with tofacitinib decreased serum levels of human IL-6 and IL-8 in the mice. However, we previously described that tofacitinib did not affect IL-6 and IL-8 production from $CD4^+$ T cells, synovial fibroblasts and $CD14^+$ monocytes *in vitro*. On the other hand, IL-17 and IFN-γ production are known to induce cytokine production from monocytes and fibroblasts and IL-6 was reported to be mainly derived from macrophages and fibroblasts of the synovium [14,15]. These findings led us to speculate that tofacitinib specifically inhibited IL-17 and IFN-γ production by $CD4^+$ T cells (presumably Th1 and Th17 cells), which in turn regulated synovitis by indirectly suppressing IL-6 and IL-8 from synovial fibroblasts and $CD14^+$ monocytes.

Furthermore, by histological evaluation, mice treated with vehicle alone, showed prominent invasion of the synovial tissue into the implanted cartilage. However, treatment with tofacitinib markedly inhibited this invasion, indicating that tofacitinib has the potential to inhibit the progress in structural damages of joints in patients with RA [MAESHIMA K *ET AL.*, UNPUBLISHED DATA].

In vivo effects of a JAK inhibitor in RA

A new concept of 'treat-to-target' is emerging for treatments of RA, whereby patients are treated according to prespecified goals, such as remission. Traditional DMARDs, most commonly methotrexate (MTX), remain the cornerstone of RA treatment. Patients who have an inadequate response to the conventional DMARDs

are often recommended to be treated with a growing number of biological DMARDs targeting TNF and IL-6, either as monotherapy or in combination with MTX. The combined use of a TNF inhibitor and MTX has produced significant improvements in clinical, structural and functional outcomes that were not previously seen and has revolutionized the treatment goal of RA to clinical remission. However, since approximately 30% of patients treated with the emerging therapy attained clinical remission, treatments in the next generation are prerequisite to patients with refractory RA [1–3]. As an orally available product with a novel mechanism of action, tofacitinib may provide a new treatment option with convenience of use and improved patient acceptance.

Kremer *et al.* reported a Phase II dose-ranging trial which was carried out to investigate the efficacy, safety and tolerability of three different dosages of oral tofacitinib in 264 patients with active RA in whom methotrexate, etanercept, infliximab or adalimumab caused an inadequate or toxic response [16,17]. Patients were randomized to four groups; placebo, 5, 15 and 30 mg of tofacitinib twice daily for 6 weeks, and were followed up for an additional 6 weeks after treatment. The American College of Rheumatology 20% improvement criteria (ACR20) response rate was 26.9. 70.5, 81.2, and 76.8% in the placebo, 5, 15 and 30 mg twice daily groups, respectively, at 6 weeks after the administration. Thus, patients treated with tofacitinib in all treatment groups were satisfied with the primary efficacy end point, ACR20 response rate at 6 weeks, significantly compared with the placebo group ($p < 0.001$). Rapid improvements in disease activity were observed in patients treated with tofacitinib, and ACR50 and ACR70 response rates significantly improved in all treatment groups by week 4. The most common adverse events reported were headaches and nausea. The infection rate in the 15 mg twice daily group and the 30 mg twice daily group was 30.4% (26.2% in the placebo group) and opportunistic infections or deaths were not observed. However, increases in mean low density lipoprotein cholesterol and high density lipoprotein cholesterol levels, and increases in mean serum creatinine level were seen in all tofacitinib treatment groups, although the mechanisms remain unknown.

A Phase II dose-ranging trial was also carried out to investigate the efficacy and safety of orally available tofacitinib in Japanese patients with active RA with an inadequate response to MTX alone [18]. A total of 140 patients were randomized to tofacitinib 1, 3, 5, 10 mg or placebo twice daily in this 12-week, double-blind, Phase II study and remained on background MTX. ACR20 response rates at week 12, a primary end point, were significant for all tofacitinib treatment groups. The ACR20 response rate was 14.3, 64.3, 77.8, 96.3 and 80.8% in the placebo, 1, 3, 5 and 10 mg twice daily groups, respectively, at 12 weeks after the administration. The ACR20 in the 5 mg group appears better than that in the 10 mg (not significant), although there are no significant background factors to explain it. Significant improvements in ACR50 and ACR70 were also obtained. Furthermore, dose-dependent increases in disease activity-score (DAS) remission rates were observed, regardless of DAS28–3 (CRP) score at baseline. In patients with high disease activity at baseline (DAS28 >5.1), the greatest percentage of patients achieving DAS remission at week 12 was observed in the tofacitinib 10 mg twice daily group (45.5%). In patients with low to moderate disease activity at baseline (DAS28 ≤5.1), the tofacitinib 5 mg twice daily group contained the greatest percentage of patients achieving DAS remission at week 12 (80.0%) (**Figure 4**).

The most commonly reported adverse events were nasopharyngitis (n = 13), and increased alanine aminotransferase (n = 12) and aspartate aminotransferase (n = 9). Incidence of common adverse events in this study appears to have different tendencyfrom those is the global Phase IIa study; decrease of hematological disorders in the study may depend on lower dose of tofacitinib (1–10 mg in Japanese study vs 5–30 mg in the global study) and increase of transaminases may be owing to the concomitant use of methotrexate in the Japanese study [16]. These adverse events were mild or moderate in severity. Serious adverse events were reported by five patients, but no deaths occurred. Taken together, in Japanese patients with active RA with inadequate response to MTX, an orally available JAK inhibitor, tofacitinib, in combination with MTX over 12 weeks was efficacious and had a manageable safety profile and tofacitinib 5 mg and 10 mg twice daily appear suitable for further evaluation to optimize their potential for the treatment of RA.

Accordingly, longer duration and dose ranging studies of this novel JAK inhibitor, tofacitinib, in the treatment of RA are ongoing by multiple global clinical examinations and efficacy for the regulation of progress in structural damages and functional disabilities is another important outcome which are examined.

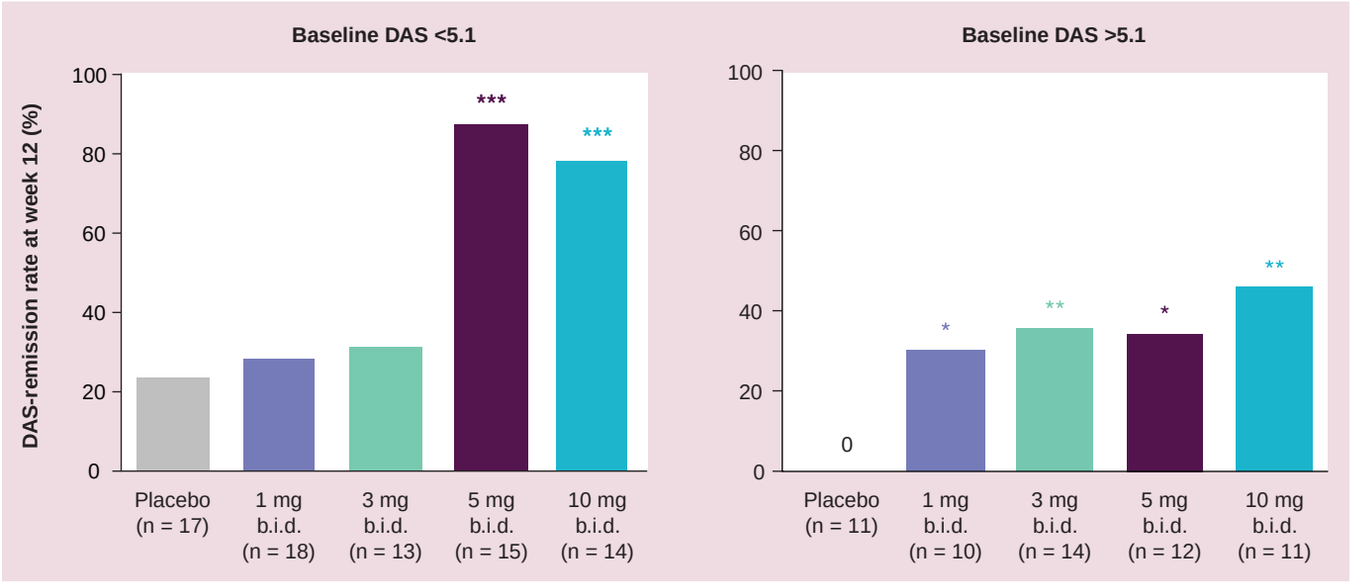

Figure 4. Phase II study of tofacitinib combined with methotrexate in patients with rheumatoid arthritis and inadequate response to methotrexate. DAS28-3 (C-reactive protein) remission (<2.6) and low disease activity state (≤3.2) rate (%) in patients with baseline DAS28-3 (C-reactive protein) ≤5.1 and >5.1 at week 12 (last observation carried forward) and treated with placebo or different dose of tofacitinib. *p < 0.05, **p < 0.01, ***p < 0.001, compared to placebo.
DAS: Disease activity score.

As for a conclusion, it has become clear that JAK inhibition with tofacitinib in patients with RA results in rapid and remarkable clinical effect equivalent to TNF inhibitors. However, we are still in the dark regarding its mechanism of action. For example, the effect of tofacitinib on joint destruction and bone metabolism is poorly understood. Our *in vitro* and *in vivo* studies have shown that tofacitinib mainly acts on T cells, subsequently suppressing cell proliferation and inflammatory cytokine production. Results of clinical trials and investigation on mechanism of action have been documented here.

Future perspective

Biological DMARDs such as TNF inhibitors have changed the paradigm of the treatment strategy of RA. Clinical remission is, however, obtained in a third of patients treated with biologics and is even sometimes experienced with antibiological product antibodies. Also, intravenous or subcutaneous administration of the drug is required and the economic issues and long-term safety remain unsolved. Accordingly, orally available low molecular weight products such as tofacitinib, targeting intracellular signaling molecules, would provide enormous power and flexibility in the treatment of RA. If tofacitinib succeeds to inhibit not only the disease activity but also joint destruction, a new paradigm will be able to emerge. Since it is possible to design low molecular weight products recognizing particular conformation of target molecules, the success in tofacitinib will facilitate the new development of multiple products, not only for RA, but also for many other inflammatory diseases.

Financial & competing interests disclosure

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Executive summary

- Tyrosine kinase is the first intracellular signaling molecule to be phosphorylated following receptor binding in a cytokine response, and 14 tyrosine kinases are known to be involved in rheumatoid arthritis (RA).
- Selective inhibition of JAK3 was considered as a potential target in the treatment of RA, and an orally available JAK3 inhibitor tofacitinib, is currently in clinical trials for RA with satisfactory effects and acceptable safety results.
- Our results suggested that the effects of tofacitinib in RA are mediated through the suppression of IL-17 and IFN-γ production as well as the proliferation of $CD4^+$ T cells without affecting synovial fibroblasts and monocytes.
- Multiple global clinical examinations indicate that JAK inhibition with tofacitinib in patients with RA results in rapid and remarkable clinical effects without severe adverse events.

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